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Effects of physiological and social challenges in different seasons on fecal testosterone and corticosterone in male domestic geese (*Anser domesticus*)

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Abstract We investigated the reliability of the non-invasive approach of measuring steroid hormones from feces in the domestic goose (*Anser domesticus*), a mainly herbivorous bird with a short gut passage time (2–3 h). Groups of eight outdoor-housed male domestic geese were subjected to three different experiments, injection of either GnRH analogue or ACTH, or "social stimulation" by confrontation with two alien males or females. These experiments were replicated in three different seasons, in spring, during peak reproductive activity, in summer, during long-day photorefractoriness and postnuptial molt, and in fall, during sexual reactivation. GnRH stimulation resulted in significant increases of mean response and peak fecal testosterone metabolites (TM) in spring and fall. Response TM concentrations excreted in spring were generally higher than in summer and fall. Social confrontation with two females, but not with two males, increased mean and peak TM in all seasons. In general, ACTH treatment resulted in a proportionally higher increase of fecal corticosterone metabolites (BM) than GnRH did in fecal TM (80- to 140-fold vs 6- to 8-fold). ACTH significantly increased mean and peak BM in all seasons. Social confrontation with two males significantly increased fecal BM in spring, but confrontation with two females increased fecal BM in fall. Our results indicate that determining steroids from feces is a generally valid approach. However, the sensitivity of the method may vary between different hormones and results may differ between seasons. BM results seemed more sensitive and seasonally robust than did TM.

Key words *Anser domesticus* · Corticosterone · Non-invasive endocrine monitoring · Steroid hormones · Testosterone

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Introduction

Steroid hormone determination from sputum, feces, or urine may be a viable alternative to blood sampling if the latter is unfeasible or undesirable. Handling, for example, causes stress and therefore interferes with measuring glucocorticoid hormones, particularly if repeated sampling is required (Miller et al. 1991).

Because of these benefits and methodological progress (Möstl et al. 1987; Palme and Möstl 1993), the non-invasive approach has gained ever-increasing importance during the past years (Wasser et al. 1993, 1997; Goymann et al. 1999). Applications include gender determination (Hurst et al. 1957; Cockrem and Rounce 1984; Bishop and Hall 1991), monitoring of the reproductive status in zoo animals and in domestic livestock (Bercovitz et al. 1982; Möstl et al. 1984; Ziegler et al. 1988; Lucas et al. 1991; Schwarzenberger et al. 1992; Hultén et al. 1995; Palme et al. 1996), or the monitoring of stress (Miller et al. 1991; Bujalska et al. 1994; Altmann et al. 1995; Creel et al. 1997; Wasser et al. 1997; Kotrschal et al. 1998). Excreted gonadal steroids may covary with social stimuli (Oliveira et al. 1996; Hirschenhauser et al. 1999a, 2000a). In addition, the non-invasive approach eases field studies (Kirkpatrick et al. 1992; Bujalska et al. 1994; White et al. 1995; Creel et al. 1996; Wasser et al. 1997; Wingfield et al. 1997; Kotrschal et al. 1998).

The use of excretion products to infer hormone status may be validated via the results obtained. For example, the seasonal patterns of fecal steroid metabolites (testosterone, corticosterone, progesterone) revealed specific relationships with gender and social status in greylag geese (*Anser anser*; Hirschenhauser et al. 1997, 1999a; Kotrschal et al. 1998). Only in a limited number of studies, however, has the non-invasive steroid determination been validated either by comparison with parallel plasma samples or by means of determining recovery rates of injected, radioactively labeled steroids, or, as in the present study, by physiological and social challenges. In general, such studies have shown that the excreted metabolites corresponded with hormone plasma titers. Peak excretion of androgen/testosterone or glucocorticoid metabolites (TM or BM) occurred in a matter of days in the case of mammals and in a matter of hours in birds (Bishop and Hall 1991; Miller et al. 1991; Wasser et al. 1993, 1997; Cockrem and Rounce 1994; Kikuchi et al. 1994; Hultén et al. 1995; Krawany 1996; Palme et al. 1996; Whitten et al. 1998; Goymann et al. 1999; Hirschenhauser et al. 2000b).

Our interest in this topic has its roots in research on the relationships between steroid hormones and social behaviour in free-ranging greylag geese. There, frequent blood sampling is unfeasible, and measuring steroid metabolites from feces may be an alternative. Recently, relationships between plasma testosterone and fecal TM were investigated (Hirschenhauser et al. 2000b). We found that GnRH challenges increased circulating testosterone and, with a time lag of approximately 2 h, also increased excreted TM. However, fecal TM was more variable than plasma TM and there were also seasonal effects on excreted androgen concentrations. At present we ask whether and how physiological and social challenges would produce responses in fecal TM and BM and how these responses would be affected by the distinct seasonality of goose reproduction.

Because controlled conditions were required, experiments were not performed with greylag geese, which are difficult to handle, but with groups of domestic geese (*Anser domesticus*) housed in outdoor pens. We expected pronounced responses to the challenges particularly during the peak of sexual activity in spring and, possibly, after the fall reactivation of the system, but less so in summer during molt and photorefractoriness (Péczely et al. 1993). Therefore, experiments were replicated during these three seasons. Groups of ganders were injected with GnRH to maximize fecal TM and with ACTH to stimulate BM excretion. In addition, non-invasive social challenges were staged by confronting groups of socially acquainted ganders with either two alien male or female geese.

Methods

All experiments were performed with outdoor-kept groups of male domestic geese (Hungarian White) at three different times of the year: March (spring peak in reproductive activity), July (summer photorefractoriness and postnuptial molt, Péczely et al. 1993), and November (fall sexual reactivation, Dittami 1981; Péczely et al. 1994; Hirschenhauser 1999a). An unmanipulated control group was available only in fall.

Ganders were housed in groups of eight in approximately 40-m^2 outdoor pens on concrete floors fenced with wire mesh at the Gödöllö Agricultural University. Birds had ad libitum access to water and food. Groups were put into their pens in February, 1 month ahead of the first experiment, and were kept within the same groups and enclosures for the rest of the year. Therefore, animals were well habituated to their situation and also to the presence of humans. Ganders were individually marked with colored plastic neck and leg rings and, in addition, with aluminum neck rings with identification numbers.

During each of the three experimental periods, two groups were injected into the pectoral muscle with 10 g superactive GnRH analogue (Ovurelin, Reanal, Hungary) per individual in the morning. To produce socially challenging situations, we confronted groups of focal males with either two alien males or females, introduced in the morning and removed again in the evening. Throughout 1 day, individual fecal samples were collected. After a 1-day interval (geese remained undisturbed), individuals of two groups of ganders were injected into the pectoral muscle with 3 IU ACTH (Richter, Hungary) in the morning.

To collect individual feces as completely as possible throughout the experimental days, the floors of the pens were covered with plastic sheeting. Each pen was continuously monitored by two observers from the outside, from approximately 7.30 a.m. to 6.00 p.m. At least 90% of the droppings were retrieved by the collectors. Samples were frozen at –20 °C within 1 h of collection.

Hormone analysis

We assayed fecal TM by enzyme immunoassay with a group-specific antibody against 17β-OH androgen (Hirschenhauser et al. 1999a,b). Because, in contrast to chickens (*Gallus gallus*; Kikuchi et al. 1994; Wallpach 1998), HPLC separation revealed that virtually all the androgen metabolites in the goose feces were conjugated (Krawany 1996), samples had to be deconjugated after extraction (below).

Fecal samples (0.5 g) were extracted with 1 ml water plus 1.5 ml methanol by vortexing (30 min). Thereafter, an aliquot of each sample was evaporated to dryness, redissolved in 500 µl acetate buffer (pH 4.8), and hydrolyzed using 500 µl of a 1:500 diluted mixture of β-glucoronidase/arylsulfatase (Merck 4114). The hydrolysis of methanol-extracted samples led to 36.0%±14.5% of unconjugated androgen. Fecal TM were then determined by enzyme immunoassay (Möstl et al. 1987) with a group-specific antibody against 4-androstene-17β-ol-3- on-carboxymethyloxine-albumin-CMO (rabbit). As label 5 α-androstane-3β, 17-diol-3-hemisuccinate (Palme and Möstl 1993) was used for biotinylation (dioxaoctane-biotin). Assay concentration limits for reliable measurements ranged from 0.4 to 30 pg/well. The specificity has been described by Hirschenhauser et al. (1999b). We assayed samples only once and determined intra- and inter-assay variations by using homogenized pool samples. The mean intra- and inter- assay coefficients of variation for the 17β-OH-androgen assay were 8.8% and 11.2%, respectively.

We assayed fecal BM by enzyme immunoassay (Möstl et al. 1987) using an antibody against corticosterone-3-CMO:BSA (working dilution 1:40,000). As a label corticosterone-3-CMO-dioxaoctane-biotin (Palme and Möstl 1993) was used (working dilution 1:200,000). The sensitivity of the assay was below 2 pg/well. For details of the procedure and cross-reactivities see Kotrschal et al. (1998). HPLC revealed that enzymatic hydrolysis with a mixture of β-glucuronidase/arylsulphatase (Merck 4114) increased the recovery of excreted H^3-labelled corticosterone from virtually zero (all metabolites conjugated) to 23%, most of which was detected by the antibody (Krawany 1996; Möstl et al., unpublished manuscript). The mean intra-assay coefficient of variation for BM was 4.2%, and the mean inter-assay coefficient of variation was 6.9%.

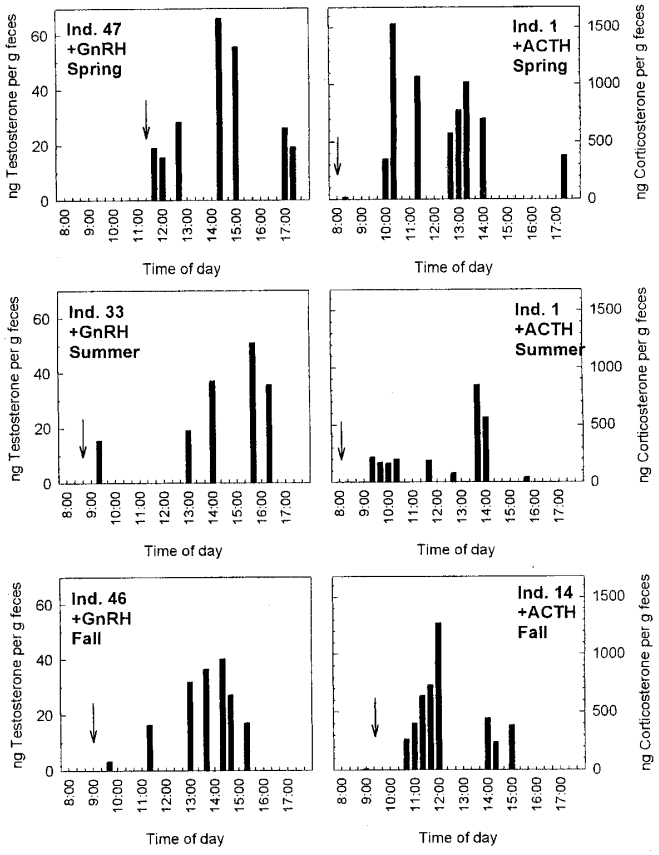

Fig. 1 Spring (*top*), summer (*middle*), and fall (*bottom*) examples of individual fecal testosterone metabolite patterns (*left column*) in response to GnRH application, and of individual fecal corticosterone-metabolites after ACTH injection (*right column*) over the course of a day. *Arrows* indicate time of challenge; each *bar* shows the value measured from a single dropping

Charcoal-marked food revealed a gut-passage time of 2–5 h (unpublished), which is in line with the literature (Mattocks 1971; Prop and Vulink 1992). Indeed, individual fecal peak values appeared during this time period (Figs. 1–3). Therefore, the individual means of samples collected within 2 h of the challenge were considered "baseline" as internal control values for the response values obtained from fecal samples collected 2–6 h following the challenge. Within season, baseline fecal TM and BM levels were compared with mean response values. In addition to mean fecal TM and BM responses (Fig. 1), baseline values were also compared with individual maxima ("peaks") of the samples collected 2–6 h after stimulation.

Because individual baseline values were rather variable, we only considered matched data pairs (where we had both baseline and response samples from the same individual within 0–6 h of an experiment). This explains the variable sample sizes (Fig. 4).

Results

Individual response patterns following GnRH or ACTH challenge

Determining hormones from feces may only be considered a valid method if GnRH or ACTH injections result in specific increases of fecal testosterone or corticosterone metabolites (TM, BM). This was indeed the case in our domestic ganders (Fig. 1). Following GnRH injection, fecal TM increased severalfold as compared to controls in spring and fall and to a lesser degree in summer. Fecal TM peaked approximately 3 h following the GnRH challenge in spring (181±66 min), but only after 6 h (350±131 min) in summer and after 3.5 h in fall (218±58 min; Figs. 1, 2).

As an early indication of the ACTH effect, defecation frequencies were enhanced and feces became more liquid. Also, the ganders showed more frequent vigilance postures when treated with ACTH as compared to GnRH (unpublished). In contrast to TM, fecal BM increased approximately 40- to 150-fold after the ACTH challenge. Peaks appeared after 4–5 h (spring: 232±135 min, summer: 298±67 min, fall: 209±66 min; Fig. 3). These peak time differences may be connected with defecation rates, which differed between seasons and treatments. Defecation frequencies of individuals over a sampling day of approximately 10 h were similar between spring and summer (4.65 times±2.84, n=60 and 3.44±2.46, n=57) but were considerably higher in fall (8.28 times±3.99, n=58). Defecation rates over the sampling day were highest in fall and were in all seasons higher after ACTH as compared to GnRH (spring: 5.9±3, n=13 vs 4±2.2, n=16; summer: 4.1±2.7, n=15 vs 1.7±1.4, n=15; fall: 8.8±4, n=12 vs 8.3±2.9, n=14).

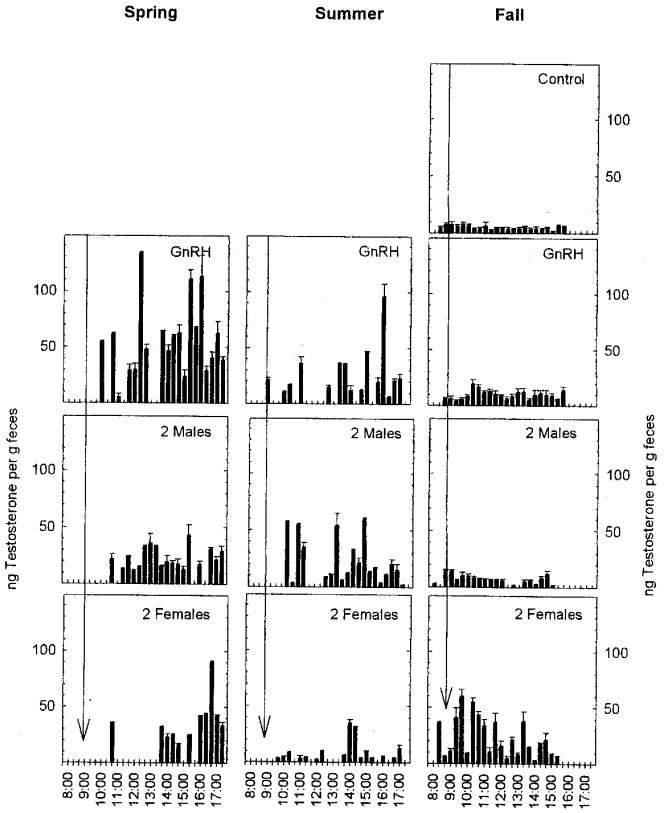

Fig. 2 Diurnal profiles of mean fecal testosterone metabolites of eight males ± SE within 20-min sampling periods in spring (*left column*), summer (*middle*), and fall (*right*). Unhandled control (top, only available in fall), following GnRH injection (*second row from top*), confrontation with two alien males (*third row from top*) and confrontation with two females (*bottom row*). Arrow indicates mean time of challenge. As individual defecation rates are irregular, the number of samples integrated per bar may vary in the range of 1–8. In bars lacking SE, n=1

Diurnal response patterns

Group profiles of mean fecal metabolites per 20-min periods over the day revealed seasonal patterns in fecal TM (Fig. 2), which were less expressed in the case of BM (Fig. 3). Following the GnRH challenge, TM was highest in spring, was lower in summer, and was at minimum in fall. However, in contrast to the other seasons, ganders in fall significantly responded to confrontation with two alien females with an increase in fecal TM. The fall group of unmanipulated controls showed a relatively flat TM profile over the day. We therefore assume that our TM patterns are responses to treatment and are not caused by an underlying circadian rhythm.

Except for the morning peak, typical for day-active vertebrates, such a low-level excretion over the day was also found for BM of the fall controls (Fig. 3). We therefore consider the peaks found over the day as responses to treatment rather than an effect of an endogenous circadian excretion regime. In response to the ACTH challenge, excreted BM was high in all seasons. Also, quite consistently between seasons, the challenge seemed to produce two to three peaks in BM over the day, one after approximately 2 h, a second one after 4 h, and possibly, a third one after 6 h. Social confrontation with two males increased BM excretion in spring but not in the other two seasons (Fig. 3, 4).

Seasonal response patterns

Seasonal group averages per treatment of baseline TM and BM from feces collected within 2 h following the challenge and of mean response and peak values from feces collected 2–6 h after the challenge (Fig. 4) substantiate the patterns of the diurnal group averages (Figs. 2, 3).

In the groups challenged by GnRH injection, baseline TM was significantly different between seasons (one-way ANOVA, F=10.455, df=2, P=0.0006). Spring and summer baseline TM were significantly higher than in fall (Bonferroni post hoc). For mean TM responses and peaks, seasonal differences were only marginally significant (F=3.182, df=2, P=0.06 and F=3.125, df=2, P=0.063). In spring, the peak significantly exceeded baseline TM (Wilcoxon, z=–2.395, n=10, P=0.017). In fall, both mean response and peak TM significantly exceeded baseline (mean response: z=–2.04, n=12, P=0.041; peak: z=–3.061, n=12, P=0.002). Fall baseline TM was not significantly different from the controls, but mean TM response exceeded the controls (Mann–Whitney U: z=–2.599, n=12/12, P=0.009 . In summer, even the peaks did not significantly exceed baseline (z=–0.73, n=4, P=0.465).

After confrontation with two alien males, TM excretion was high in all three seasons (Fig. 2) but did not

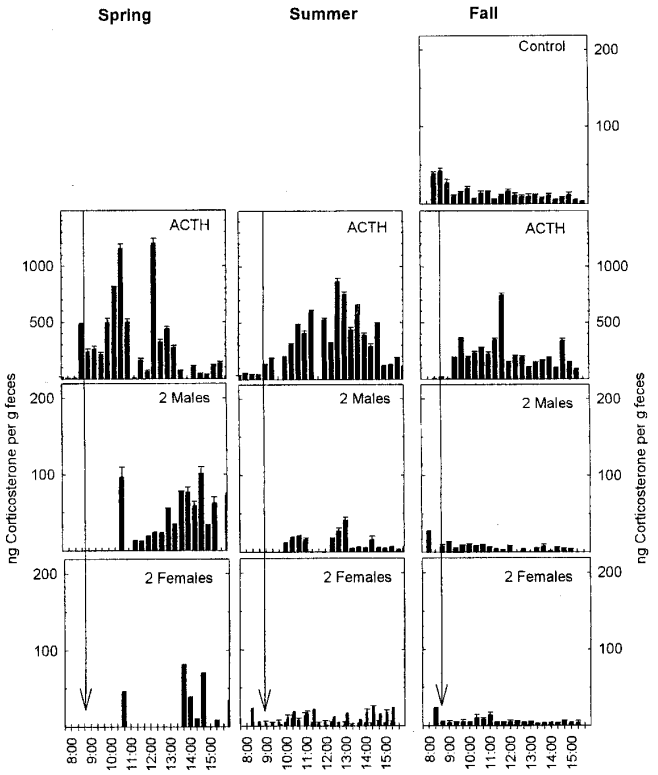

Fig. 3 Diurnal profiles of mean fecal corticosterone metabolites of eight males ± SE within 20-min sampling periods in spring (*left column*), summer (*middle*), and fall (*right column*). Unhandled control (*top*, only available in fall), following ACTH injection (*second row from top*), confrontation with two alien males (*third row from top*), and confrontation with two females (*bottom row*). Average time of challenge shown by the arrow. As individual defecation rates are irregular, the number of samples integrated per bar varies in the range of 1–8. In bars lacking SE, n=1

reach significance thresholds (Fig. 4). Baseline TM and peaks were not significantly different between seasons, but response TM values were marginally so (one-way ANOVA, F=3.793, df=2, P=0.06; Bonferroni testing revealed no significant differences between any two groups). Neither in spring nor in summer were mean response and peak TM values significantly different from baselines. In fall, however, response TM was significantly higher than baseline or control (Mann–Whitney U: z=–2.004, n=4/12, P=0.045), but also baseline was significantly higher than the unmanipulated control TM (z=–2.488, n=4/12, P=0.013), probably because the presence of the two alien males had already increased fecal TM within the 2 h of baseline sampling (Fig. 2).

Except for fall, response TM and peaks were not significantly different between seasons, but baseline TM in the groups challenged with two alien females was (Fig. 4; one-way ANOVA, F=4.592, df=2, P=0.036; Bonferroni testing revealed a higher baseline in spring as compared to summer). Neither in spring nor in summer were mean response and peak TM levels significantly different from baseline. In fall, however, peak TM (z=–2.201, n=6, P=0.028) and also mean response TM were significantly higher than baseline (Mann–Whitney U: z=–3.374, n=6/12, P=0.001).

The ACTH challenge produced proportionally much larger responses and peaks of excreted BM than GnRH did with TM (Fig. 4). In all three seasons mean responses and peaks of BM significantly exceeded baseline or the unmanipulated controls (available only in fall). Neither baseline BM values nor response or peak values differed significantly between seasons. However, in all seasons, differences between baseline and both response and peak BM were marginally significant (Wilcoxon, spring baseline vs response and peak: z=–1.825, n=4, P=0.068; summer baseline vs mean response: z=–2.803, n=10, P=0.005; summer baseline vs peak: z=–2.52, n=8, P=0.012; fall baseline vs mean response and baseline vs peak: z=–2.023, n=5, P=0.043).

Confrontation with two alien males produced a significant increase of peak BM only in spring (Fig. 4; ANOVA, with Bonferroni post hoc test: response: F=14.597, df=2, P=0.0011; peak: F=26.396, df=2, P=0.0001) and only in spring did the peak exceed baseline with marginal significance (Wilcoxon: z=–1.826, n=4, P=0.068). Still, in all three seasons, mean response and peak fecal B values tended to be higher than baseline. Baseline BM was not significantly different between seasons.

In the male group confrontated with two females, baseline, response, and peak BM were significantly higher in spring as compared to summer or fall (Fig. 4; ANOVA, with Bonferroni post hoc test: baseline: F=19.38, df=2, P=0.0002; response: F=11.216, df=2, P=0.002; peak: F=22.656, df=2, P=0.0002). No significant differences were found between baselines, respons-

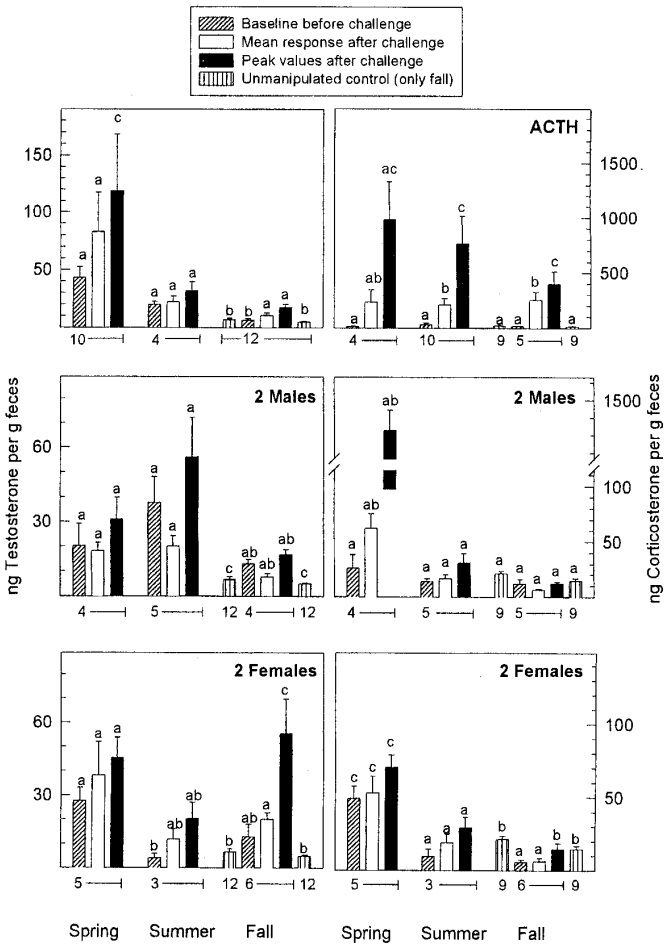

Fig. 4 A seasonal comparison (means + SE) of fecal baseline, mean response, and peak testosterone (TM) and corticosterone metabolites (BM). Stimulation with GnRH, ACTH, and with either two alien males or two alien females (baseline sampled 0–2 h after stimulation, response sampled 2–6 h after the challenge). An unmanipulated control group was only available in fall. Two groups of eight ganders were available per season and treatment. Sample sizes vary (*n* shown at *x*-axes) because individuals were included only when both baseline and response values were available (mainly depending on defecation, which varied between seasons). Significant differences between baseline and response as well as between seasons indicated by different letters (*a–c*)

es, and peaks in spring or summer. In fall, the peak, but not the mean response, was significantly higher than baseline BM (Wilcoxon: z=–2.201, n=6, P=0.028). However, in fall BM of the unhandled controls were also significantly higher than either baseline or response BM (Wilcoxon, both: z=–2.201, n=6, P=0.028).

Discussion

Our results, by and large, confirm that the steroid status can be non-invasively inferred from feces. However, results have to be interpreted with caution. Treatments with GnRH and ACTH as well as social stimulation had the expected effects on fecal TM and BM, and BM responded in a more pronounced way to a releaser challenge than did TM. But there was also an unexpected effect of season, at least on TM (Figs. 1–4), probably for two major reasons: changes in circulating hormones due to the seasonality of reproduction and seasonal changes in clearance.

Seasonality of reproduction explains highest plasma T and highest responsiveness to stimulation in spring (Dittami 1981; Hirschenhauser 1999a,2000a; Hirschenhauser et al., unpublished manuscript). In summer, during peak refractoriness and molt, fecal BM but not TM could be stimulated by our treatments (Fig. 4). In fall, TM could be stimulated by GnRH, and, to a quite striking extent, by social confrontation with two alien females (Figs. 2–4). These results are in line with findings that social changes may promote responses in steroid hormones (Wingfield et al. 1990; Oliveira et al. 1996; Hirschenhauser et al. 1997, 2000a; Kotrschal et al. 1998; Silverin 1998). The fact that social confrontation with two alien males caused the highest increase of excreted BM in spring (Fig. 3) may reflect seasonal alertness and competitiveness in connection with reproduction. In summer, physiology is determined by photorefractoriness (Dittami 1981; Péczely et al. 1993) and is functionally tuned towards molt and predator avoidance: at least it was in the wild ancestors of domestic geese, which should still behave according to evolutionary history. Accordingly, social confrontation had hardly an effect on TM or BM in summer (Figs. 2–4). In fall, the system resumes sexual and social responsiveness in

preparation for the spring reproductive period (Wingfield and Farner 1980), and our challenges had the predicted effects on fecal TM and BM, however, of smaller magnitude than in spring.

In contrast to fecal TM after GnRH stimulation, there was no significant seasonal effect on excreted BM following stimulation with ACTH (Figs. 2–4), even though summer and fall BM excretion regimes seemed lower than in spring. In the light of biological functions this is to be expected. Corticosterone is part of the general stress response, which is not restricted to the reproductive season. Predation and environmental perturbations (Wingfield et al. 1997) occur year-round and individuals need to remain responsive to all kinds of stressors. Thus, the caveats concerning potential seasonal effects on TM excretion may not apply to the circannual non-invasive monitoring of stress (Kotrschal et al. 1998).

The high variability of fecal metabolite values within and between individuals (Figs. 1–4) necessitates considerable sample sizes. This may also explain why a number of trends in this study remained non-significant (see TM, for example, in Fig. 4). A higher variability of fecal steroid metabolites as compared to their plasma values (Hirschenhauser et al. 2000b) may be explained by a considerably longer chain of physiological events from stimulation to the fecal sample measured with fecal samples as compared to plasma. From plasma into feces, steroids are metabolized in the liver (Tailor 1971) and are excreted via the gut and/or kidneys, which necessarily increases variability. Additional variability is added by the assay procedure, for example, by deconjugation, which was necessary, because in contrast to domestic fowl (Cockrem and Rounce 1994; Wallpach 1998), virtually all excreted steroids were found to be conjugated in geese (Krawany 1996).

However, seasonal trends, and variabilities, are probably not caused by qualitative shifts in excreted metabolites and therefore, the substrate the antibody can bind to. Correlations of results from the same samples analysed with the present antibody against 17β-OH androgens and a novel, epiandrosterone-antibody against 17-oxo groups including the 17β-OH androgens (Möstl, unpublished manuscript) were highly significant (Hirschenhauser et al. 2000b).

A contribution to the high variability of steroid metabolites in the droppings my have come from the unseparable urine component. For BM, Helton and Holmes (1973; mallard *Anas platyrhynchos*) as well as Krawany (1996; domestic goose) concluded that the hepato-renal pathway may even be more important than the hepato-intestinal one. Significant interspecific differences in the time course and pathway of excretion, via urine or feces, were found in a number of species (Kikuchi et al. 1994; Peter et al. 1996; Whitten et al. 1998). Peak urine excretion of infused ^{14}C-cortisol, for example, occurred. after approximately 12 h in sheep (*Ovis domesticus*), after 24 h in ponies (*Equus caballus*) and only after 48 h in pigs (*Sus scrofa domestica*; Palme et al. 1996). In bighorn sheep (*Ovis canadensis*), the urine cortisol peak appeared only 2–6 h after an ACTH challenge (Miller et al. 1991). In baboons (*Papio cynocephalus*; Wasser et al. 1993) as well as in sows (Hultén et al. 1995) and sheep (Palme et al. 1996), the time lag between blood progesterone and its peak appearance in feces was 1–2 days. In an owl (*Strix occidentalis caurina*), ACTH produced a fecal B peak after 12 h (Wasser et al. 1997).

Gut-passage time is certainly important for the length of the time lag between plasma peaks and metabolites appearing in feces. In domestic geese, where gut-passage time is 2–5 h, the temporal resolution of the method is potentially good. There may be seasonal changes in clearance from the blood system and also seasonal effects on retention times of gut contents, affecting how fast hormone metabolites that entered the gut via the bile appear in the feces. In barnacle geese (*Branta leucopsis*; Prop and Vulink 1992), for example, food retention time was negatively correlated with the amount of food ingested and varied between 1.9 h in winter, 4.2 h during molt, and 7.9 h during incubation. In domestic geese, peak radioactivity appeared within 2 h after ^{14}C-corticosterone application (Krawany 1996). In this herbivorous bird, urea accounts for only a minor proportion of the dropping (8.2% in barnacle geese, Prop and Vulink 1992). Because urea could not be separated, our measurements actually integrate both urinary and fecal excretion pathways. This is feasible, since the fast gut passage counteracts the potentially long time lag between fast urinary excretion and slow excretion in feces caused by slow gut passage in most mammals.

Still another source of variability may be the short-term-oscillatory diurnal excretion pattern found particularly for BM (Fig. 3). The mechanism of oscillatory excretion, particularly of BM, is unclear. This could be caused by the rhythmic activity of the gut. If the metabolites from the liver are secreted into the gut at a constant rate via the *Ductus choledocus*, and, for some reason, intestinal activity is rhythmical over the day, this could result in the present pattern. Another possibility is that the oscillatory excretion pattern reflects the rhythmical activity of the liver-bile system caused by feedback regulation.

In sum, our data from domestic geese indicate that the non-invasive approach may yield valid results, if comparisons of sex steroids between seasons or reproductive states are done with caution. Seasonal effects, for example, may affect TM responses and to a lesser extent, BM excretion.

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